Proposed title for the Ph. D. work: Study of chromium toxicity in Zebrafish

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Introduction:

Chromium is a grey hard metal commonly found in the trivalent state in nature. In the natural environment it occurs predominantly in two valencies as Cr [VI] and Cr [III] out of which the former is regarded as toxic. Chromium is a heavy metal belonging to Period- 4 and Group- 6 of Periodic Table with Atomic Number- 24 and Atomic Weight- 51.996. The three most stable forms in which chromium occurs in the environment are the 0 (metal and alloys), +3 (trivalent chromium), and +6 (hexavalent chromium) valence states. In the +6 valence state, chromium exists as oxo species such as CrO_3 and CrO_4^{2-} that are strongly oxidizing. Cr [VI] can exist in aquatic media as a water-soluble complex anion and may persist in water for long periods. Cr [VI] is a moderately strong oxidizing agent and reacts with organic matter or other reducing agents to form Cr [III]. Naturally occurring chromium is usually present as Cr [III]. Cr [VI] in the environment is almost totally derived from human activities (WHO, 1990).

Cr [VI] is one of the most common contaminants found in surface and ground water. Welding, grinding, and polishing of stainless steel are the principal sources of chromium pollution, while the other sources include burning of fossil fuels and waste incineration. The most important industrial sources of chromium in the atmosphere originate from ferrochrome production. Ore refining, chemical and refractory processing, cement-producing plants, automobile brake lining and catalytic converters for automobiles, leather tanneries, and chrome pigments also contribute to the atmospheric burden of chromium (Fishbein et al.,1976). Chromate chemicals used as mist inhibitors in cooling towers and the mist formed during chrome plating are probably the primary sources of Cr [VI] emitted as mists in the atmosphere.

Relevance of study

Chromium compounds, such as Cr [VI], are widely used in electroplating, stainless steel production, leather tanning, textile manufacturing, and in wood preservation which results in the release of aqueous chromium to the subsurface at numerous sites. The disposal of waste leads to Cr [VI] contamination of the rivers, lakes which chronically affects the flora and fauna including fish and subsequently accumulate at higher trophic levels, possibly due to food chain magnification. Hexavalent chromium [Cr (VI)] is found to be hazardous to health(Das et al., 2008). It has been

also found to be related with bronchogenic cancer(Halasova et al., 2009).A number of Chromium (VI) compounds have been demonstrated to be mutagenic or clastogenic. Elevated prevalence of both chromosomal aberrations and sister chromatid exchange in lymphocytes are found in workers exposed to chromates(Jelmert et al., 2005).

According to OSHA (Occupational Safety & Health Administration) the *Permissible exposure limit (PEL)* in air for chromium [VI] should not be more than 5 micrograms per cubic meter of air (5 μgm^{-3}), calculated as an 8-hour time-weighted average (TWA). Chromium [VI] is listed by the Environmental Protection Agency as one of the 129 priority pollutants and one of the 14 most noxious heavy metals. The recommended limit for Cr [VI] concentration in water is 1 $\mu g\, L^{-1}$ for Cr [VI]. In the effluents in the vicinity of Cr industries the levels of Cr range from 2 to 5 $g\, L^{-1}$ (Chandra et al., 1997).

Levels above permissible limit have also been found at various residential areas. Cr [VI] content in surface water near a dichromate factory in Durres, Albania, was found to be around 131.68 mgL^{-1} (Lazo et al., 2009) whereas it is suggested that gastrointestinal effects in humans may occur at an exposure level of 20 mgL^{-1} (Zhang et al.,1987).

The aquatic ecosystems in Ranaghat–Fulia region are reported to be heavily contaminated by Cr, which originate from the handloom textile factories operating in the area and study also indicates possible human health hazards through consumption of Cr contaminated fish. Most of the average daily dietary Cr intake estimates representing various populations living in different countries range between 30 and 60 µg (Krejpcioet al., 2001), while the Estimated Safe And Adequate Daily Dietary Intake (ESADDI) of Cr as proved by the Food and Nutrition Board of the US National Academy of Science in 1989 for adult man is 50–20 µg /day (Anderson et al., 1992).

Thus hexavalent chromium (Cr [VI]) contamination is regarded as a major occupational hazard, found above the permissible limit in chromium related industrial areas. So studying the toxicity of Cr [VI] is of high relevance.

Objectives:

1. **To determine the LC_{50} of chromium (VI) in zebrafish (*Danio rerio).***
2. **To study hepatotoxicity and expression pattern of antioxidant biomarker genes in order to assay chromium induced oxidative stress in zebrafish.**
3. **Expression pattern of Nrf2/Keap1 in the liver tissue after Chromium exposure.**
4. **To study Chromium induced cell death in zebrafish hepatocytes.**

Toxicity Studies of Cr [VI] in zebrafish (*Danio rerio*)

Toxicity test of chemicals on animals has been used for a long time to detect the potential hazards posed by chemicals to man. Fishes have been extensively used as test organisms for acute toxicity bioassays due to several advantages. According to U.S. Environmental Protection Agency, they are easily maintained under laboratory conditions, and they are sensitive to a variety of pollutants as well as generally available throughout the year from both commercial and natural sources. Among freshwater animals, zebrafish, *Danio rerio* are widely used for vertebrate embryo toxicity bioassays. Evidence has long suggested that zebrafish is a good model to predict toxicity of various compounds that are toxic to humans. It is now widely used in many research areas due to several advantages like easy availability, low maintenance cost and breeding in laboratory condition as well as similarity with human genome (Howe et al., 2013)

In vivo studies will be done on zebrafish treated to sub lethal concentration of potassium chromate (K_2CrO_4) subsequent to determination of LC_{50}.

Detecting acute toxic effects of chromium

DETERMINE THE LETHAL CONCENTRATION (LC_{50}) OF CHROMIUM:

The potential toxicity of chemical substances often is presented as their LC_{50}. LC_{50} is the concentration of a substance that is lethal to 50% of the organisms in a toxicity test. LC_{50} can be determined for any exposure time, but the most common exposure period is 96 hours.

To address this issue, 10 fish will be maintained in a glass tubs filled with 1L of testing water and exposed to different concentrations of Cr [VI]. The mortality will be recorded every 24 h till completion of 96 h. The concentration that will kill 50% of the experimental animals (zebrafish) will be recorded as the LC_{50}.

HISTOLOGICAL ANALYSIS TO DETECT ALTERATION IN TISSUE ULTRASTRUCTURE:

Histological study is an integrated parameter, providing a more complete evaluation of the organism's health, effectively mirroring the effects of exposure to a particular toxicant.

To address this issue, at the end of acute exposure time (96 h) fish of all groups will be sacrificed. Tissues from control and treated fish will be dissected out, fixed in Bouin's Fixative**.** After 24 h of fixation the tissues will be subjected to series of ethanol concentration for dehydration followed by embedding in a paraffin block. it will be cut into sections of 6 µm onto a glass slide and stained with hematoxylin-eosin for histological assessment according to Bancroft et al. (1996). Observation of the prepared slides under light microscope may reveal some changes encountered due to chromium toxicity in fish tissue.

Detecting chronic effects of chromium

Adult zebrafish will be exposed to $1/10^{th}$concentration of $LC_{50}$for 90 days. Subsequently on 15^{th}, 30^{th}, 60^{th} and 90^{th} day, fish will be sacrificed and tissue will be collected for further toxicological assays.

DETECTION OF OXIDATIVE STRESS MARKERS IN ORDER TO ASSAY CHROMIUM INDUCED HEPATOTOXICITY:

The liver plays a central role in transforming and clearing xenobiotics and is susceptible to the hence induced toxicity. Study of chromium induced hepatotoxicity will be done.

Cr [VI], due to its redox potential can induce oxidative stress in fish and impair their health. The antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione related enzymes protects organisms from the toxic effects of the activated oxygen species and helps to maintain cellular homeostasis by removing ROS like superoxide anion, hydrogen peroxide, and hydroxyl radical. These biomarkers when assayed will give an account of the induced oxidative stress.

Following assays will be done to assess the oxidative stress.

- **Quantification of lipid peroxidation.**

The process of Lipid peroxidation (a well-defined mechanism of cellular damage)results in reactive compounds such as Malondialdehyde (MDA).

Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage. These aldehydic secondary products of lipid peroxidation are generally

accepted markers of oxidative stress. Thiobarbituric Acid Reactive Substances (TBARS) is a well-established assay for screening and monitoring lipid peroxidation. MDA forms a 1:2 adduct with thiobarbituric acid. The MDA-TBA adduct formed from the reaction of MDA in samples with TBA can be measured colorimetrically or fluorometrically. TBARS levels are determined from a Malondialdehyde equivalence standard (Yagi et al., 1998;Lef'evre et.al., 1994).

To address this issue, hepatic tissue will be collected after dissection of both treated and control fish. The tissue after being homogenised will be used for TBARS Assay. 1 ml of sample solution will be mixed with 2 ml of TBA- TCA- HCl and subsequently heated for 15 minutes at 95°C. After a brief incubation, the absorption of pink colored supernatant will be determined at 535 nm.

- **Estimation of reduced Glutathione (GSH):**

Thereduced glutathione will be estimated by following the method of Ellman et al., 1961. 0.5 ml of tissue homogenate will be mixed with 2.5 ml of DTNB (5, 5 dithiobis- 2- nitrobenzoic) solution. After 5 min of incubation, the intensity of yellow colored solution will be spectrophotometrically determined at 405 nm.

- **Biochemical assays and colorimetric detection of enzymatic activities of the oxidative stress markers like catalase, super oxide dismutase etc.**

Catalase activity assay:Catalase is an antioxidant enzyme omnipresent in eukaryotes that destroys hydrogen peroxide by dismutation. The enzyme is concentrated in the peroxisome subcellular organelles.

Catalase Activity Assay will be done from liver homogenate according to Aebi et al., 1984. According to the protocol, Assay buffer containing tris- HCl, hydrogen peroxide and EDTA will be added to liver homogenate. The Catalase in the tissue homogenate will decomposethe hydrogen peroxide (H_2O_2) into water and oxygen. The rate of disintegration of hydrogen peroxide into water and oxygen is proportional to the concentration of catalase. After the time at which the reaction is proceeded for exactly one minute, the catalase will be quenched with Sodium azide. The remaining hydrogen peroxide in the reaction mixture facilitates the coupling reaction of DHBS and AAP in conjunction with an HRP catalyst. The quinoneimine dye coupling product thusformed will be measured at 520nm, which correlates to the amount of hydrogen peroxide remaining in the reaction mixture. Thus more the catalase activity less the quinoneimine dye concentration.

Superoxide Dismutase Assay: Superoxide dismutase (SOD), one of the most important antioxidative enzymes, catalyzes the dismutation of the superoxide anion into hydrogen peroxide

and molecular oxygen. To detect the presence of oxidative stress in hepatic tissue, SOD will be analyzed in liver.

SOD activity will be determined according to the method of Beauchamp and Fridovich (Beauchamp et al., 1971). To address this issue, at first tissue homogenate will be prepared from hepatic tissues of both treated and untreated fishes. The tissue lysate supernatant will be collected and will be used for the assay as sample.

This assay of superoxide dismutase is indirect and depends upon its ability to scavenge Superoxide anions(O_2^-) from reaction mixtures and thus to inhibit reactions caused by O_2^-. Superoxide anions are generated by a Xanthine/Xanthine Oxidase (XOD) system, and then detected with a Chromagen Solution. However, in the presence of SOD, these superoxide anion concentrations are reduced, yielding less colorimetric signal. On addition of Xanthine Oxidase Solution, color is developed. The more SOD activity, the less O_2^- left for Xanthine oxidase to use, hence less the color.

STUDY OF EXPRESSION PATTERN OF ANTIOXIDANT GENES AS BIOMARKERS.

Cell has a battery of antioxidant proteins that get expressed under stress condition. The expression patterns of antioxidant genes such as GST, CAT, Cu/ Zn SOD, Mn/ SOD and GPX will be studied at both mRNA levels and protein levels by means of Quantitative Real Time PCR and western blotting technique.

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EXPRESSION PATTERN OF Nrf2 AND Keap 1 IN CHROMIUM TREATED CELLS.

The transcription factor, known as nuclear factor (erythroid–derived 2)- like 2, has been recognized as the master regulator of cellular defense mechanism against toxic insults.It is responsible to induce the transcriptional expression of phase II detoxifying enzymes such as glutathione S transferase (GST), CAT and SOD. Nrf2 remains anchored to actin bound Kelch-like ECH-associated protein 1 (Keap1) or inhibitor of Nrf2 (INrf2), which negatively regulates Nrf2 through ubiquitination and proteasomal degradation in the cytoplasm (Itoh et al., 1999). However, in response to ROS, Nrf2 is released from Keap1, translocates to the nuclei and transactivates the constitutive induction of detoxifying enzymes along with oxidative stress proteins. The transcriptional expression of Nrf2 is enhanced under stress condition.

The transcriptional pattern of Nrf2 and Keap1 will be studied by means of Quantitative Real Time PCR. Total RNA extracted from tissue of zebrafish will be subjected to RT- PCR for formation of cDNA which will be subjected to quantitative Real Time PCR for amplification. The level of amplified DNA detected by the help of Fluorescent reporter probes corresponds to the initial level of RNA, thus giving an account of expression pattern of a particular gene.

The protein levels of Nrf2 and Keap 1 will also be observed by means of western blotting. Study of Nrf2 and keap 1 expression at mRNA and protein levels will help inunderstanding the possible involvement of Nrf2/ Keap 1 pathway in chromium induced toxicity.

CELL VIABILITY ASSAYS IN CELLS FROM ZEBRAFISH LIVER

Single cell suspension of liver will be prepared from liver of both treated and non treated zebrafish. The cell suspension will be subjected to following cell viability assays .

Trypan blue assay:

The cell suspension prepared from liver tissue will be simply mixed with trypan blue dye and visually examined with the help of haemocytometer. Living cells possess intact cell membranes that extrude trypan blue dye, where as dead cell do not.

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium reduction assay:

To address this issue cell suspension is prepared from liver tissue, which is then subjected to treatment with MTT. Viable cells with active metabolism convert MTT into a purple colored formazan product. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured at 595 nm using a plate reading spectrophotometer **(**Mosmann et al., 1983; Marshall et al., 1995). Percentage of viable cells will be calculated taking the viability of the untreated cells as 100%.

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